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OTDK

Effect of electrical microstimulation
parameters on in vivo neuronal calcium
responses in the visual cortex of anesthetized
mice

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Abstract

Sensory neuroprostheses, such as visual and auditory implants, aim to reconnect individuals with lost sensory experiences by delivering precisely targeted electrical stimulation through implanted neural interfaces. These cutting-edge technologies strive to bridge the gap between artificial and natural perception, offering a pathway to restore and even enhance sensory function.

Yet, challenges remain in understanding the precise neuronal activation patterns elicited by electrical stimuli of neuroprostheses. Despite widespread use, electrical microstimulation lacks comprehensive insights into the spatio-temporal patterns of neuronal activity it evokes. Our research project aims to address this knowledge gap by employing flexible multi-shank neural probes for electrical stimulation which contain iridium-oxide microelectrodes precisely inserted into layer 2/3 of the primary visual cortex (V1) of mice. In vivo calcium imaging in Thy1-GCaMP6 transgenic mice makes it possible to investigate the spatio-temporal effects of advanced electrical microstimulation patterns of varying stimulation parameters, including current amplitude, stimulation frequency, and pulse duration on cortical activity. Beyond this, it allows to study neuronal activations to more sophisticated stimulation patterns, such as dynamic current steering. A custom high-channel-count neural stimulator was used to generate the stimulation patterns, and a two-photon laser scanning microscope (wavelength: 900 nm; raster scanning at 31 Hz) with a 20x water immersion objective (field of view: 550 μm x 550 μm) to capture neuronal calcium responses.

Our preliminary findings indicate that increasing stimulation current and pulse duration markedly enhances both the number of activated neurons and the spatial spread of activation in case of monopolar stimulation, while increasing the stimulation frequency, though activating more neurons, has a weaker effect. Additionally, we studied the temporal activation of neurons between the two stimulating electrodes using dynamic current steering.

Observed differences in response patterns suggest that adjusting stimulation parameters can finely tune neuronal activation in space and time, offering potential improvements for neural prostheses. Our results provide insights into electrical microstimulation-evoked activity in V1 of mice, with future work focusing on optimizing stimulation strategies to improve the spatial resolution and efficacy of cortical prostheses for visual restoration.

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Chapter 1.

Introduction

In summary, all great work is the fruit of patience and perseverance, combined with tenacious concentration on a subject over a period of months or years.

—Santiago Ramón y Cajal

Vision plays a fundamental role in human experience, providing a sense of spatial awareness, emotional connection, and independence. Vision impairment, including blindness, is a significant global health issue, with severe personal and socioeconomic impacts. A Global Burden of Disease study conducted in 2019 identified vision impairment, including blindness, as the third-leading cause of disability-adjusted life years, underscoring the significant impact of vision loss on quality of life worldwide [1]. In 2020, approximately 1.1 billion people were affected by vision loss globally, with 43 million individuals blind, 295 million have moderate to severe visual impairment, 258 million people have mild visual and 510 million people have near vision problems [2]. The economic impact is also substantial, with annual global productivity losses due to vision impairment estimated at 410.7 billion USD [3]. Furthermore, with an ageing global population and the increasing prevalence of diabetes both major contributors to vision loss the demand for effective treatments, such as advanced prosthetics and targeted therapies, is expected to rise, aiming to enhance the quality of life for millions and reduce both the healthcare and economic burdens of vision loss worldwide.

Efforts to address vision loss have led to the development of various therapeutic options, ranging from pharmacological treatments and surgical interventions to advanced technologies like sensory neuroprostheses. For individuals with complete vision loss, one of the most promising solutions lies in the field of visual neuroprostheses, which encompasses devices designed to restore some level of sight through electrical stimulation of a region in the visual pathway. Current visual prosthetic technologies offer hope for restoring basic visual perception, enabling blind individuals to regain partial sight [4]. However, these devices come with significant limitations. Retinal implants, for example, are limited by the spatial resolution they can achieve due to the density of electrodes and the constraints of current microstimulation techniques [5]. Cortical implants stimulate the visual cortex directly and show promising results but face challenges such as electrode durability, immune response, and limited capacity to replicate the complexity of natural vision [6].

Recent advances in flexible electrode technology and advanced simulation techniques offer potential improvements to the above-mentioned issues. Flexible polymerbased probes are better suited to integrate with neural tissue, minimizing tissue damage and reducing the immune response. Additionally, stimulation techniques like current steering allow more precise control of activation patterns across electrodes, enhancing the spatial resolution of stimulation. While technological advancements are essential, the pressing challenge remains the understanding of the precise spatio-temporal patterns of neuronal activation triggered by electrical microstimulation and how these elicited patterns compare to neural responses evoked by natural visual stimuli. Acquiring comprehensive knowledge about this matter will aid us to construct a system capable of restoring sensory experiences that are as close as possible to, or potentially better than, their natural functional state [7]. Our research is part of the HYPERSTIM¹ Horizon Europe project funded by the European Innovation Council's Pathfinder Challenge program, aiming to improve the effectiveness and resolution of visual prostheses for the blind by developing advanced stimulation patterns. Within this framework, our study specifically focuses on investigating intracortical electrical microstimulation-induced neuronal activity patterns in the visual cortex of anesthetized mice using in vivo calcium imaging. By using penetrating, flexible electrodes and two-photon microscopy, our main objective is to examine how different electrical microstimulation parameters such as amplitude, pulse duration and frequency-affect neuronal activation. Our specific objectives include (1) examining how these parameters modulate the spatial and temporal patterns of neural activation and (2) assessing the feasibility of using complex stimulation techniques, such as current steering and current focusing, to achieve more controlled and localized neuronal responses. By investigating how variations in stimulation parameters affect neuronal responses in the visual cortex, we hope to uncover insights that could pave the way for more effective visual prostheses with improved spatial resolution.

¹ <https://hyperstim.eu/>

Chapter 2.

Related works

The eye houses the biological machinery that takes in light rays and converts them to neural signals. These signals carry visual information and pass through a complex and intricately designed pathway within the central nervous system, enabling an accurate representation of the surrounding environment. The obtained visual information is evaluated by a process called visual perception, which is performed by a complex network of interconnecting neurons that form the visual system.

2.1. Introduction to the visual system

The process of vision in the primate visual system begins when light enters the eye and is transformed into electrical signals by photosensitive chemical reactions of photoreceptor cells located in the retina. Light enters the eye through the lens and the cornea, which both act to refract the light onto the retina, which is the innermost coat of the organ, as well as a neural layer. The neural retina contains five types of cells: the photoreceptor cells (the rods and cones), the horizontal cells, the bipolar cells, the amacrine cells, and the retinal ganglion cells [8]. In the primate eye, two major types of retinal ganglion cells can be found. Namely, type M and type P cells, which process information about different stimulus properties. Type P retinal ganglion cells outnumber the M-ganglion cells and are colour-sensitive object detectors which produce a slowly adapting response. Type M retinal ganglion cells are much larger with bigger receptive fields and are colour-sensitive motion detectors [9].

The central area of the retina, called the fovea, has densely packed cone photoreceptors resulting in high visual acuity. Photoreceptor cells transform photons of light into electrical signals through the process of phototransduction, then transfer this signal to bipolar cells, which in turn synapse with ganglion cells, which transmit the signal from the eye [10]. Thus, the direct pathway for the transmission of visual information from the eye to the brain includes only the receptor cells, bipolar cells and ganglion cells making them the first three cells in the visual pathway. The horizontal and amacrine cells function as modulators of synaptic activity, thereby affecting the transmission of visual information [8]. From the retina, signals exit through the axons of the retinal ganglion cells that form the optic nerve. Most axons then synapse in the layers of the lateral geniculate nucleus (LGN) of the thalamus. The left and right sections of the

LGN correspond to the areas of the retina in each eye responsible for perceiving the right and left visual fields, respectively. Like the retina, this structure is also laminated, consisting of six principal cell layers [8]. The largest cells form the deepest two layers numbered 1 and 2 (called magnocellular layers) and receive projections from the type M cells within the retina and consist of mLGN cells, which behave like M-retinal ganglion cells. Smaller cells form four layers above them (layers 3-6, called parvocellular layers) and receive projections from the type P ganglion cells. Interposed between these principal laminae are the thin koniocellular layers of the smallest cells receiving projections from type P ganglion cells as well [11].

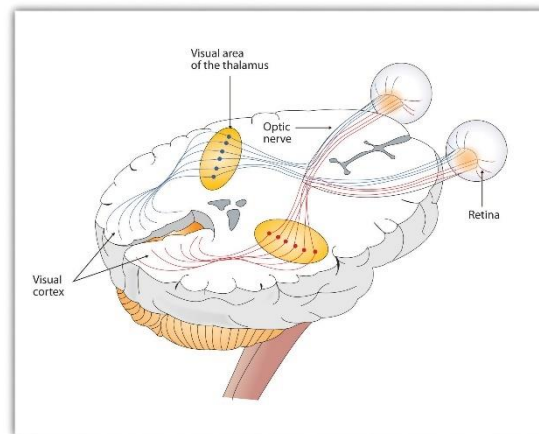


Figure 2.1: The pathway of visual information travels through the thalamus and into the visual cortex, with each eye transmitting visual information to both the left and right hemispheres of the brain. Source: [12]

The optic tract fibres from each eye synapse in different layers of the LGN. The input from each eye is handled separately, nerve fibres derived from the contralateral eye (opposite side of the body) terminate on cell bodies in layers 1, 4 and 6, while those of the ipsilateral eye (uncrossed) terminate in layers 2, 3 and 5 [13]. Thus, the LGN in each hemisphere receives information from both retinæ, but each LGN neuron is monocular, meaning it responds to stimulation of one eye only.

The LGN sends its fibres via the optic radiation to the occipital lobe which is the visual processing area of the neocortex. It includes Brodmann areas 17, 18 and 19, forming the visual cortices. Brodmann area 17 or V1 form the primary visual cortex, while V2, V3 and V4 (Brodmann areas 18 and 19) form the higher visual areas [14].

The primary visual cortex, also known as the striate cortex, gets its name from a distinctive white layer of myelinated fibers, called the stria of Gennari, which characterizes this region. V1 is mainly located on the medial surface of both brain hemispheres along the calcarine sulcus, with much of it lying deep within this fissure that extends from the parieto-occipital sulcus to the back of the occipital lobe [9].

Structurally, V1 is organised into horizontal layers and vertical columns. Layer 1 is the

most superficial layer containing few scattered neurons. Neurons in layer 2 send axons only to deeper cortical layers, while in layer 3 neurons communicate with both near and far cortical locations [9]. Layer 4 contains the stria of Gennari and is divided into two main sublayers, one of which processes input from the magnocellular layers of the LGN (layer 4C α), while another from the parvocellular layers (layer 4C β) [15]. Layer 4 sends axons to more superficial parts of the visual cortex and other visual cortical areas. Layer 5 sends axons to the superior colliculus and other areas in the brainstem, while layer 6 sends projections back to the LGN.

As for the vertical columns, neurons with similar receptive fields are arranged into columns approximately 0.5 mm in diameter. These columns can be further subdivided into smaller units which respond to specific attributes of visual information such as orientation, colour, and ocular dominance [9]. These columns are absent in the cortical area representing the physiological blind spot, as this region only receives input from one eye. The visual cortex supports functions such as contour analysis and binocular vision, relying on both its horizontal and vertical organization. The cells within V1 are primarily activated by input from the LGN, though additional input comes from other cortical areas. V1 interacts with the superior colliculus and frontal eye fields and is essential for the initial processing of all visual information needed for visual perception. Damage to this area leads to vision loss in the opposite visual field (contralesional hemifield) [11].

The axons of the different types of LGN neurons terminate in different layers or sublayers of the primary visual cortex. The topographic (spatial) organization of retinal neurons is preserved across the entire visual system. This means, that the myelinated fibres entering the primary visual cortex maintain their segregation preserved from the retina and LGN. This organisation is known as retinotopy which refers to the two-dimensional mapping of visual input from the retina onto the target structure [15]. Consequently, neighbouring parts of the retina project to neighbouring parts of the LGN which then project to neighbouring parts of V1. This retinotopic organization in the visual pathway carries significance because it allows the receptive field of a cell in the visual system to be defined as the specific area of the retina (or visual field) that can affect that cells firing [15].

The higher order visual areas surrounding V1 (i.e., V2, V3 and V4) are thought to each contribute uniquely to the activity of the primary visual cortex, refining its processing capabilities. V2 is heavily involved in color processing, with dense clusters of color-selective neurons and a complex retinotopic organization that supports detailed spatial mapping and high-resolution imaging, especially in foveal regions. V3 focuses on orientation and motion processing, crucial for dynamic scene interpretation, with neurons that respond to both color

and direction. V4 adds higher-level processing, such as form and shape perception, which aids in object recognition by enhancing sensitivity to complex contours [16].

Feedback from these areas enhances V1s responsiveness by amplifying weaker sensory signals, especially for stimuli within a neurons receptive field, though this comes at the cost of reduced selectivity to orientation and direction. Altogether, feedback from higher order visual areas selectively strengthens weak V1 responses, potentially enabling more robust processing of visual stimuli [17].

2.1.1 Differences in the visual system of primates and rodents

During our work we conducted experiments in the primary visual cortex of mice, therefore it is important to note a few main differences between the primate and a rodent visual system.

Rodents are not capable of performing cognitive tasks at the same complexity as primates. Additionally, primates rely heavily on vision for interacting with their environment, while mice are capable of using visual cues and depend more on other senses (e.g., touch). Consequently, primates have an advanced visual system with a complex hierarchy of cortical areas specialized for processing diverse visual information. In comparison, mice have simpler visual cortex with fewer hierarchical levels [18]. Moreover, research indicates that mice and primates use distinct methods for visual segmentation, with mice showing limitations in segmenting visual scenes- their visual system is more regionally homogeneous-compared to primates [19].

2.2. Vision restoration strategies

A better understanding of the intricacies of the visual system has led researchers to develop targeted strategies for vision restoration including optical aids, gene therapies, and neural interfaces [20]. By integrating both technological and biological approaches, vision restoration strategies aim to provide those with visual impairments the ability to perceive light, shapes, and, in some cases, basic details of their environment [10].

Although many advances have been made in recent years regarding the identification of pathological mechanisms underlying visual diseases, there is yet to be an effective treatment for many patients who suffered severe degeneration or damage to their visual system.

Since the initial studies involving electrical stimulation of the eye to explore vision restoration methods, notable progress has been made in the fields of neuroimaging, electrophysiology, and bioelectrical interfaces. These developments have allowed researchers to examine the structure and function of the visual pathway in greater detail and to identify potential therapeutic targets for vision restoration.

At present, a diverse range of visual restoration strategies including gene or stem cell therapy and optogenetic stimulation have been proposed and are being investigated [21].

2.2.1 Gene therapy

Gene therapy, for example, is possible for those with inherited retinal degeneration caused by recessive mutations [22]. Currently, clinical trials of gene therapy (i.e., gene replacement or augmentation strategy) are limited to conditions where genetic mutations lead to a lack of a functional gene product, excluding dominant diseases [23].

2.2.1.1 Optogenetics

Optogenetic methods aim to genetically modify nerve cells with viral carriers to grant photosensitivity to the remaining retinal cells, after which specific wavelengths can modify neuronal activity [24]. Like gene therapy, optogenetics can only be used with patients in the early stages of degenerative diseases. Additionally, optogenetic approaches are being explored not only in the retina but also in the cerebral cortex, aiming to restore visual function by directly modulating cortical neurons [25].

2.2.2 Stem cell therapy

In contrast to the gene-specific gene therapy, stem cell therapy could be useful for individuals with significant cell damage in their retina because the treatment does not need a significant number of healthy cells to be successful. Stem cell therapy introduces new, healthy cells that can differentiate and potentially replace damaged retinal cells, providing structural and functional support. This approach is particularly promising for advanced stages of retinal degeneration, where it can potentially restore light sensitivity and visual function, as described by Uy and colleagues (2013) [26].

2.2.3 Electrode-based visual prosthesis

Neural interfaces are advanced biomedical systems that create a direct link between the nervous system and external devices, and are designed to record, interpret and even stimulate neural activity. These devices allow real-time monitoring of neural signals and precise modulation of neural activity, thus playing a key role in brain-computer interfaces, neuroprostheses, and neuromodulation therapies [27]. In visual applications, stimulation can evoke neuronal activity by delivering current to the targeted neural tissue, resulting in phosphene-perceived visual sensations within the patients visual field. These phosphenes are typically consistent in size and shape when stimulation is repeated with the same parameters.

In recent years, neural interfaces have been used to restore lost motor functions and improve sensory processing (e.g. some cochlear implants have enabled individuals with severe hearing loss to perceive sound by directly stimulating the auditory nerve) [28] and diagnose or treat neurological disorders like epilepsy and Parkinson's disease [29]. Neural interfaces can be invasive, with electrodes implanted within neural tissue, or non-invasive, with sensors positioned outside the body. Another important application of neural interfaces is the visual prosthesis, which aims to restore some degree of sight by artificially reactivating downstream visual neurons using electrical stimulation, thus bypassing the damaged segment in the visual system [4].

2.2.3.1 Retinal prosthesis

A retinal prosthesis can be used in patients with retinal diseases that have resulted in the loss of photoreceptors. Many neural implants target the retina because of the relative simplicity of the interface and ease of surgical implantation of the device. Several types of retinal implants exist, with subretinal and epiretinal implants comprising a significant portion of retinal prosthesis development [30]. A notable example of an epiretinal implant is the Argus II Retinal Prosthesis System, which received FDA approval in 2013 [31]. This device consists of 60 electrodes and has been implanted in over 350 patients worldwide. Another device is the Alpha AMS which is a subretinal implant featuring 1600 electrodes that offer higher spatial resolution [32].

The fundamental operation of these devices involves the stimulation of the remaining photoreceptors in the retina with electric signals. However, retinal prosthesis can only work if there is still a functional neural link to the visual system. The fragility of the retina also brings up challenges [4].

2.2.3.2 Optic nerve-based prosthesis

If the retina has already reached a high level of deterioration due to retinal disease, but the optic nerve is still intact, it can be a potential target for vision restoration. Evoked potentials in the visual cortex with electrical stimulation via optic nerve implants show waveshapes similar to the ones obtained in normal-sighted people during visual stimulation [33]. Furthermore, prior research has noted a decrease in stimulation threshold over time. Yet, high stimulation amplitude is required to elicit a low-resolution perception, and it is important to remember that this method can only work if the nerve is intact [4]. An example device would be the Artificial Vision by Direct Optic Nerve Electrode (AV-DONE) system which uses electrodes on the optic disc. In an initial trial with one human volunteer, the subject reported localized phosphene

perception in over 50% of tests, though the electrode array often became dislodged. The improved design has 7 stimulation and one reference electrode to aid stability [34].

2.2.3.3 Thalamic visual prosthesis

Ninety percent of retinal ganglion axons synapse at the lateral geniculate nucleus, which makes it a potentially good access point to implant a visual prosthesis for individuals with either retinal or optic nerve damage [4]. The receptive fields of LGN neurons are straightforward, well-defined, and resemble those found in the retina, therefore a small number of LGN neurons could be sufficient to achieve a simple perception with stimulation [35]. Additionally, the receptive fields in the LGN maintain a uniform spatial density across the visual field, holding this as an advantage over the retina where receptive field density varies. Thalamic visual prosthesis would need multiple electrodes to produce distinct phosphenes. Due to the complex organization of the LGN, each electrode must precisely target small, specific areas to create clear, spatially accurate phosphenes. Achieving precise electrode placement and controlled stimulation remains a technical challenge, as improper activation could lead to blurred or overlapping phosphenes. Current research suggests that approximately 500 phosphenes are needed to provide reasonably useful vision. Ongoing research includes the development of a prototype device with 64 electrodes implanted on both sides (for a total of 128 stimulation sites) in monkeys [14].

2.2.3.4 Cortical implants

V1 was the main focus of many early functional studies, including electrically induced phosphenes. This is because most visual information is initially channelled through V1 before travelling to higher areas of the visual cortex [15]. The key advantage of cortical prostheses is their placement downstream, which provides rehabilitation opportunities for blind and visually impaired individuals for whom retinal, optic nerve or LGN visual prostheses would not be effective [4]. Additionally, cortical implants allow for the longest period of therapeutic intervention. Rather than complete neural degeneration, post-injury compensatory plasticity mechanisms engage deafferented neurons from surrounding cortical areas, enabling stimulation long after the injury or disease has occurred. Another benefit of cortical implants comes from the larger surface area, which reduces the need for miniaturization compared to the implants mentioned above. This larger area allows for the implantation of more electrodes, potentially enhancing resolution and improving visual outcomes [6].

One limitation of the cortical approach, however, is the complexity of the representation of visual information. For example, based on the location of the stimulating electrode, the evoked

percepts could be quite different, therefore another challenge is the large outspread of V1. Additionally, the foveal portion of V1 in humans (representing the central part of visual space) is sometimes found buried in the calcarine fissure which can complicate the implantation process [35].

Similar to the LGN, the vertical columns in the primary visual cortex exhibit a relatively uniform spatial density across the surface. This is a direct consequence of cortical magnification, which allocates a larger area of the cortex to the central visual field that corresponds to high-acuity vision [36]. This consistent spatial density allows the columns in V1 to facilitate a more reliable transmission of visual information. In V1 stimulation, both surface implants (placed on the cortical surface) and intracortical implants (penetrating deeper into cortical layers) can be used. Stimulating multiple sites through current injection results in the perception of multiple phosphenes [15].

2.2.4 Basic structure and operation mechanism of electrode-based vision restoration systems

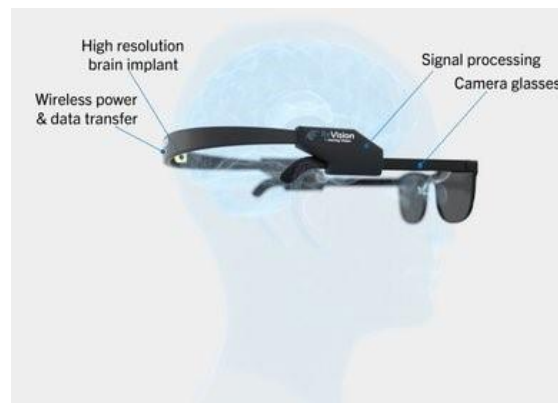


Figure 2.2: In general, a visual prosthesis contains: A Camera: often integrated into an eyeglass frame. An Implant: microelectrode arrays implanted in the nervous tissue for stimulation. External electronics: to transform the video stream of the camera into a stimulation pattern and to communicate to the implant. Source: [37]

An electrode-based vision restoration system typically involves a small camera mounted on glasses or a headband to capture visual information. This image data is sent to a video processing unit (VPU) that decimates and converts it into a sequence of commands determining stimulation parameters. These commands are then wirelessly transmitted to the implanted device, where patterned electrical stimulation activates neurons around specific electrodes, eliciting visual perception.

The wireless transmission overcomes risks associated with transcutaneous wires (infections and severe scarring) [6][4].

2.3. Neural interfaces to measure and modulate brain electrical activity

Neurophysiology is a fundamental technique in neuroscience, enabling the study of electrical activity within the nervous system. Measuring the electrical signals generated by neurons and other excitable cells offers a direct window into the communication processes of the brain. Monitoring voltage changes, action potentials and synaptic events provide essential information to understand how neural circuits operate, a comprehension vital to study brain functions and disorders [38][39].

The most commonly used electrophysiological technique is the extracellular recording, where an electrode is placed near a neuron allowing precise investigation of its electrical activity with high spatial (on the micrometer scale) and high temporal resolution (on the millisecond scale) without penetrating the membrane. In the domain of extracellular recordings, intracortical multi-electrode arrays are used to measure single-cell activity (single-unit activity) or the collective electrical activity recorded from a small population of neurons near the electrode (multi-unit activity). The multi-electrode arrays can be used in both acute and chronic experiments. Moreover, these neural probes can be used not only for measurements of electrical activity, but also for electrical stimulation to modulate the activity of neurons [40].

2.3.1 Electrode architectures

Devices used for neurophysiological applications differ widely in their level of invasiveness, which is often directly related to their specific purpose. For example, electrodes designed to be placed on the scalp can capture slow fluctuations of brain waves at frequencies of approximately 0.1 Hz to 100 Hz. In contrast, implantable electrodes are designed to be able to detect single-unit activities at frequencies from 0.2 to 7 kHz [4].

Therefore, probe design and materials are critical components in the development of neural implants, this is particularly true for chronic applications. Flexible polymer substrates are becoming increasingly popular due to their ability to minimize mechanical mismatch (differences in mechanical properties like stiffness and flexibility between two materials in contact) and reduce stress between the probe and its surrounding tissue. The most commonly used flexible substrates for intracortical electrodes include parylene, polyimide and SU-8 [39]. Besides the materials, the dimensions of the probe are also of key importance. Minimizing the thickness and width of neuroprosthetic implants (i.e., their surgical footprint) is essential for enhancing their flexibility and promoting better integration with the brain tissue. Reducing these dimensions means the implant can match the mechanical properties of the surrounding tissue more closely, reducing stress between the probe and the tissue [16].

2.3.2 Electrical microstimulation

The fundamental mechanism of electrical stimulation is based on the communication of neurons through electrical impulses known as action potentials, which occur when the voltage difference between a neurons intracellular and extracellular spaces becomes more positive. This process is facilitated by voltage-gated ion channels that allow ions to move once the membrane potential surpasses a threshold, leading to cell depolarization [41]. When an external cathodic stimulus pulse is applied, it negatively charges the extracellular space relative to the intracellular space, initiating an action potential. Electrical stimulation can thus influence neural activity by altering the membrane potential. The use of square-wave pulses in neuronal stimulation helps control the timing of impulses and regulate cell activity [42].

Injecting electrical current with a multi-electrode array targeting the surrounding neuronal tissue, therefore, can induce action potentials that may be utilised to restore various specific functions in the brain. In our experiments, stimulating the visual cortex may result in the perception of phosphenes [43].

Techniques for stimulating visual responses in the brain can be broadly classified into invasive and non-invasive methods. Non-invasive techniques include transcranial electrical stimulation, which stimulates the brain through the scalp. Although these approaches avoid the risks which can arise from surgical implantations, the response characteristics of brain activity and the elicited phosphene sizes lack precision.

In contrast, invasive techniques include subdural electrodes positioned on the cortical surface and intracortical microelectrodes which penetrate the cortical layers. The intracortical approach offers the highest precision, as it allows closer proximity to individual neurons within the visual cortex, resulting in lower stimulation thresholds for evoking phosphenes [16].

2.3.2.1 Cortical microstimulation

Unlike retinal or thalamic prostheses, which rely on stimulating earlier stages in the visual pathway, cortical microstimulation bypasses damaged areas of the eye and optic nerve, directly engaging the brains higher-order visual processing centres. This method has the potential to provide visual information to individuals with damage to multiple levels of the visual pathway. However, a major challenge with cortical microstimulation is ensuring that each electrode produces clear and distinct phosphenes [43]. Expanding on cortical microstimulation, researchers have investigated various methods to stimulate neurons within the visual cortex, specifically through surface and intracortical microstimulation techniques [44].

Surface stimulation

Epicortical prostheses are less invasive since it does not require the penetration of the brain. On the flip side, the current amplitude needed to elicit phosphene is much higher than with intracortical approaches (in the range of a few mA) [43]. This is the consequence of the distance between the electrode and the targeted neurons. This distance reduces the spatial resolution of artificial vision. Additionally, the higher current amplitudes can cause seizures and therefore be unsafe.

Intracortical stimulation

While this approach is more invasive, the close contact between the electrodes and the neurons enables intracortical microstimulations (ICMS), a more selective stimulation with much lower current amplitudes (in the range of a few μA) [45]. This results in a smaller evoked phosphenes, closer to the stimulated location in the retinotopic map. Despite its widespread use, ICMS is yet to be understood in terms of the spatio-temporal patterns of evoked neuronal activity and how this relates to neural responses triggered by visual stimuli. In our project, we use penetrating flexible electrodes specifically to investigate these evoked neuronal patterns, aiming to gain deeper insights into ICMS-evoked neural responses [41].

The limitation of this approach is the inability to place the stimulating electrodes in the interhemispheric fissure, where a notable part of the visual field is mapped in humans. For this reason, stimulation can only evoke phosphenes in a few degrees of the visual field. A solution to this issue could be to place the electrodes in higher-order visual areas where spatial representation is still preserved [40].

In ICMS, several key parameters can be adjusted to achieve and optimize precise neural activation. Current amplitude in ICMS typically ranges from 1 to 100 μA , as lower currents effectively stimulate target neurons while reducing tissue damage [45]. Pulse duration (or pulse width) commonly falls between 100 and 500 μs , where shorter durations mean less charge delivery to the surrounding tissue. Frequency, (the number of pulses per second), generally ranges from 10 to 200 Hz, depending on the desired neural response [46]. Finally, ICMS often uses chargebalanced biphasic pulses, alternating between cathodic and anodic phases, to prevent electrode polarization and reduce tissue damage. Together, these adjustable parameters provide a high degree of control over neural activation [47][48].

2.3.2.2 Advanced stimulation strategies

In the pursuit of restoring vision, advanced stimulation strategies have become critical. Traditional visual prosthesis, while groundbreaking, face limitations in resolution and

specificity, as their fixed array of electrodes cannot effectively mimic natural visual experiences. To overcome these challenges, researchers are developing more refined methods to deliver information to the brain. The importance of electrode architecture has already been mentioned above, this section is meant to highlight the central role of stimulation techniques in creating a more detailed and dynamic visual perception.

One stimulating strategy is current focusing, which aims to stimulate narrower, more specific regions of neural tissue by applying current across multiple electrodes simultaneously, shaping the electric field. By stimulating with multiple electrodes the electric field created will be more concentrated resulting in a reduced current spread and more localised neural activation. In cochlear implants, this technique often through tripolar configurations allows for precise targeting of auditory nerve fibres [49].

Another approach within these advanced strategies is current steering, a technique that allows for more precise control over stimulation patterns, potentially increasing the spatial resolution of visual prostheses without increasing the physical number of electrodes [7]. This technique works by manipulating the distribution of electrical currents across adjacent electrodes of a multielectrode array, creating "virtual" stimulation points (virtual electrodes) between the physical electrodes [50]. By adjusting the intensity and ratio of current sent to neighboring electrodes, it is possible to generate intermediate activation sites, effectively increasing the spatial resolution of the stimulation field. With dynamic current steering, which works by varying the current ratios dynamically, current steering can finely shift the location of neural activation, allowing for more precise targeting of neural tissue [51]. This advanced technique enables prosthetic devices to deliver more continuous sensory information with higher resolution [52].

Current steering has been successfully implemented in cochlear implants to enhance pitch perception by creating intermediate frequencies between fixed electrodes [47].

Similarly, in deep brain stimulation, it has improved the selectivity and efficacy of stimulation by shaping the electric field within neural tissue [53].

Chapter 3.

Methods

3.1. Neural probe design

In our study, we used flexible polymer-based brain-implantable devices designed and fabricated by ReVision Implant (KU Leuven, Leuven, Belgium)², a startup company with whom we collaborate in the HYPERSTIM project. These probes have three shanks, each one is 1 mm long with platinum wiring inside and contains 7 iridium-oxide microelectrodes per shank used for electrical stimulation. The microelectrodes have an average electrical impedance of approximately 50 k Ω (Fig. 3.1). The widest inter-shank distance (distance between the edges of the shanks) is 200 μ m. Each shank ends in a sharp tip to aid implantation and one microelectrode is positioned at the tip of each shank, with the other electrodes arranged in pairs at varying distances (350 μ m, 500 μ m and 650 μ m) from the tip. The distance between neighbouring electrodes is 20 μ m and the area of the rectangle-shaped electrodes is 20 μ m x 30 μ m (Fig. 3.1). The probe is connected to the neurostimulator device (developed by ReVision Implant) via an adapter printed circuit board.

3.2. Animal surgery and probe implantation

In the acute experiments presented here, we worked with transgenic mice with genetically encoded intracellular calcium indicators, namely C57BL/6J-Tg(Thy1-GCaMP6s)GP4.3Dkim/J (hereafter GCaMP6s) and C57BL/6JTg(Thy1-GCaMP6f)GP5.17Dkim/J (hereafter GCaMP6f) [54]. Data were obtained from three adult GCaMP6f (two female, one male) and two GCaMP6s (one male, one female) mice and an additional GCaMP6f(male) animal for our experiment performed with dynamic current steering. The average weight of the mice was 33.4 g. The animals were anaesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally (IP) by weight, then regularly updated (1-2 injections/hour) with the same mixture to maintain the level of surgical anaesthesia. The physiological body temperature of the animals was maintained with the help of a homeothermic heating pad placed under them. The heating pad equipped with internal temperature sensors was controlled by a temperature control unit (Supertech, Pécs, Hungary). The surgery starts with placing the mouse into a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and after exposing the skull, a rectangular craniotomy (~4 mm x 3 mm) centred over the visual cortex is drilled (the

² <https://revision-implant.com/>

starting point of the craniotomy is located 0.5 mm posterior to the bregma and 0.5 mm lateral to the midline).

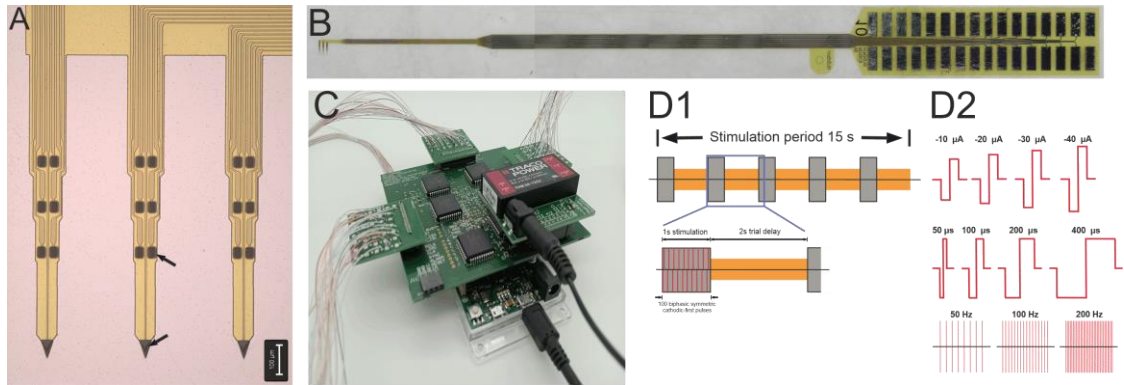


Figure 3.1: (A-B) Polyimide high-density electrode arrays used in the experiments have shanks with a cross-section of $20 \times 70 \mu\text{m}^2$ (thickness $\times$ width). The shanks are $1000 \mu\text{m}$ long and are spaced at a fixed distance of $250 \mu\text{m}$ from each other. Rectangular iridium oxide electrodes (indicated by arrows) ($20 \times 30 \mu\text{m}^2$) are arranged along the width and length of each shank, forming a grid with spacing ranging from $15 \mu\text{m}$ to several $100 \mu\text{m}$. (C) The neurostimulator device used to generate pulse trains for electrical stimulation. (D1) Schematic representation of our standard electrical microstimulation protocol. (D2) The different stimulation parameters changed during the experiments.

A glass coverslip with a diameter of 3 mm (Multi Channel Systems, Reutlingen, Germany) is then placed in the anterior part of the craniotomy, which is then fixed with light-curing dental cement (RelyX Universal Resin Cement, 3M, St. Paul, MN, USA). The following step is the implantation of the flexible polymerbased probe at a slow, consistent insertion speed (approximately $2 \mu\text{m/s}$) at an angle of $\sim 55^\circ$ with the help of a motorized stereotaxic micromanipulator (Robot Stereotaxic, Neurostar, Tübingen, Germany) (Fig. 3.2). The aim is to reach layer 2/3 of the primary visual cortex (V1). After the insertion, a biocompatible silicone (Dura-Gel, Cambridge Neurotech, Cambridge, United Kingdom) is dropped onto the still-exposed part of the cortex for protection and probe stabilization. Finally, a small drop of dental cement was applied to secure the probe cable to the mouse's skull. Throughout the surgery, room-temperature physiological saline solution was periodically dripped into the craniotomy to keep the exposed brain tissue hydrated until the Dura-Gel was applied (Fig. 3.2).

3.3. Two-photon microscopy

During the experiments, a two-photon laser scanning microscope was used to observe cortical activity in the primary visual cortex (V1) of transgenic mice. Two photon microscopy is a specialized fluorescence microscopy technique that enables high-resolution and high-sensitivity fluorescence detection even in vivo in brain tissue, where photons are strongly scattered. This property is utilized with transgenic mice containing the GCaMP6 gene, which

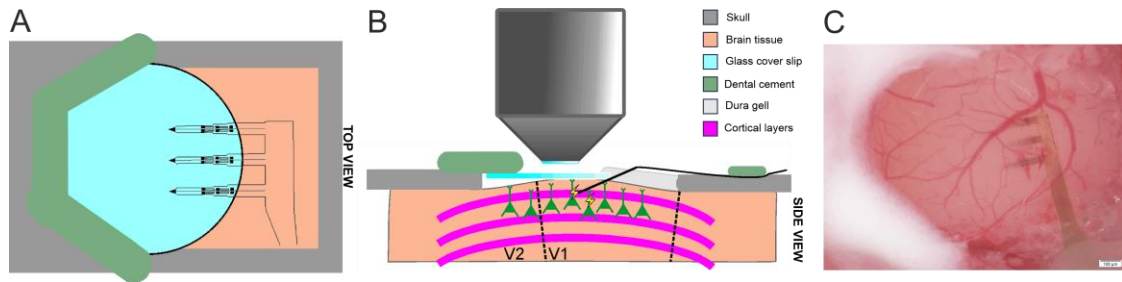


Figure 3.2: (A) A schematic representation of the rectangular craniotomy (~4 mm x 3mm) is prepared, centered over the visual cortex (V1) of GCaMP6 transgenic mice anaesthetized with ketamine/xylazine. A glass coverslip with a 3 mm diameter is then carefully placed in the anterior part of the craniotomy and fixed to the skull using a small amount of light-curing dental cement. (B) A two-photon laser scanning microscope with a 20x water immersion objective was used to image calcium activity in layer 2/3 of the visual cortex adjacent to the probe. (C) Realised craniotomy with the implanted probe.

expresses the GCaMP6 fluorescent indicator. This indicator exhibits increased fluorescence when the intracellular calcium concentration elevates in neurons.

In two-photon microscopy, two low-energy photons, in the near-infrared or infrared wavelength range, cooperate to create a transition in the fluorescent molecule between the ground and excited states. Therefore, two photons are absorbed simultaneously by the molecule being excited, and their energies are combined. At the focal point of the infrared laser pulses in the microscope, such light intensity is achieved that, in turn, causes the fluorescent dyes, which absorb in the visible range, to be excited. With this method, the fluorescent dye or indicator molecule in the sample emits visible light at roughly twice the frequency after absorbing infrared light. Due to the deeper penetration of infrared light into the tissue, cells can be examined even hundreds of micrometres deep.

This two-photon effect must occur within a femtosecond time window, which requires ultrafast lasers with short pulses (80-120 fs) that contain high photon density. The imaging itself is performed with a scanning procedure, where mirrors allow the excitation spot to move quickly across the examined surface, scanning the focal plane, which is then arranged by computer software.

The advantages of two-photon microscopy include the deeper penetration of the light beam into tissues, as infrared light scatters and absorbs less in the sample. This enables the examination of deeper structures and neurons in living tissues. Moreover, in two-photon microscopy, unlike confocal microscopy, the fluorescent signal originates only from the activated areas, and no fluorescent signal comes from the surrounding inactive areas. This results in reduced phototoxicity, which is less disruptive to living samples. Two-photon microscopy (Femto2D two-photon laser scanning microscope system, Femtonics, Budapest, Hungary) with a 20x water immersion objective (Olympus XLUMPLFLN) with a 2mm

working distance and a numerical aperture of 1.0, providing a field of view (FOV) of $550\ \mu\text{m}$ x $550\ \mu\text{m}$ was used to perform calcium imaging. Frames (images) with a size of 512×512 pixels were collected by raster scanning at a frame rate of ~ 31 Hz. A Coherent Chameleon Ultra Ti:Sapphire femtosecond laser tuned to a wavelength of 900 nm was used for calcium imaging. After placing the mouse under the objective, first, the location of the probe shanks and microelectrodes were identified, then cortical microstimulation was started.

3.3.1 Calcium imaging

Calcium indicators used for two-photon calcium imaging can be either chemically or genetically encoded. Genetically encoded calcium indicators are more advantageous for two-photon calcium imaging because they can target specific cell types and can be used for weeks or even months in chronic experiments. For this reason, the indicator used in the current series of experiments is also a genetically encoded calcium indicator, specifically the GCaMP6f (fast) and GCaMP6s (slow) types.

GCaMP6 indicators, due to their high sensitivity and rapid decay kinetics, are suitable for monitoring neuronal impulses or synapses and represent a significant advancement in understanding neuronal activity in living organisms. The slower GCaMP6s variants are more sensitive to small changes in calcium ion levels, while the faster GCaMP6f variants are less sensitive but have faster kinetics. Their structure consists of a calmodulin-binding M13 peptide, a circularly permuted green fluorescent protein (GFP), and calmodulin.

Calcium imaging is made possible by the influx of calcium ions into the intracellular space of a neuron when an action potential occurs, leading to an increase in ion concentration. At this point, calcium ions bind to the calcium-sensing molecule, calmodulin, which undergoes a conformational change and tightly binds to M13, preventing water molecules from accessing the circularly permuted GFP, which fluoresces brightly in its anionic form [55].

3.4. Electrical microstimulation

In our experiments, we conducted electrical microstimulations with custom developed Arduino and Python-based software, paired with a neurostimulator device (provided by ReVision Implant) to generate pulse trains. During electrical stimulation, current flows between the electrodes (between the active electrode(s) and the ground) through the brain.

Each square-wave pulse rapidly rises to a certain value, holds that value for a specified time, and then suddenly drops back to the baseline. The duration during which a pulse exerts its effect is referred to as the pulse width, while frequency denotes the number of pulses delivered per second (Hz). To avoid tissue damage, we used a current between $\pm 1\ \mu\text{A}$ and $\pm 50\ \mu\text{A}$. The

primary aim of our electrical stimulation experiments was to examine the impact of various basic stimulation parameters on visual cortical activity, including current amplitude, pulse duration, and stimulation frequency. These modifications in the stimulation parameters have been made possible by the adjustable Python code.

Our standard parameter set for stimulation includes 100 biphasic, symmetric cathodic-first pulses (200 pulses for GCaMP6s mice) with a duration of 200 μ s per phase and with a current amplitude of -40 μ A. Pulses were delivered at 100 Hz frequency (200 Hz for GCaMP6s mice), resulting in approximately 1 second long stimulation trials. Each stimulation was repeated five times (five trials) with a 2-second delay between trials, resulting in a total stimulation period of approximately 15 seconds. Prior to the stimulation, we recorded a $\sim$ 10 seconds long pre-stimulation period, and after completing the trials, we captured a short post-stimulation period. We applied monopolar stimulation with a single active electrode. The other microelectrodes on the probe served as ground electrodes during stimulation. To examine the impact of specific stimulation parameters on neuronal activity, we only changed the parameter under investigation in each test (Table 3.1).

For our experiments with dynamic current steering, we stimulate neurons between two electrodes during which the amplitude of the stimulation pulses changes in time (decreases on one electrode and increases on the other). Here, we applied dynamic current steering between two tip electrodes of the three-shank flexible probe with 200 biphasic, symmetric cathodic-first pulses of 200 μ s pulse duration per phase generated at 200 Hz. The patterns of activation were observed for the two tested stimulation currents (-40 and -50 μ A) and were repeated three times consecutively. The recordings obtained from these stimulation sessions were saved and processed offline for later analysis.

3.5. Processing and analysis of calcium imaging data

Suite2P is a Python-based end-to-end pipeline for processing two-photon calcium imaging recordings. The input files to the Suite2p processing pipeline were our two photon recordings converted into TIFF files. I concatenated multiple recordings (e.g. recording with the same parameter set) into a single file to improve the detection of single neurons. The Suite2p pipeline consists of four stages: image registration, ROI (region of interest) detection, quality control, and activity and neuropil extraction [56].

The output of this process is multiple .npy files containing all the information collected throughout the processing. It stores the ROIs and neuropil traces and whether each ROI is a cell or not. The information about each ROI is also extracted and can be used to recover the

corresponding pixels for each ROI. In the graphical user interface (GUI) the ROIs can be seen as masks over each detected cell and instances not classified as cells.

Parameter set variables				
GCaMP6f				
Examined variable	Current amplitude (μ A)	Pulse duration (μ s)	Stimulation frequency (Hz)	Pulse number
Current amplitude	-10, -20, -30, -40	100	100	100
Pulse duration	-40	50, 100, 200, 400	100	100
Stimulation frequency	-40	100	50, 100, 200	100
GCaMP6s				
Current amplitude	-10, -20, -30, -40	100	200	200
Pulse duration	-20	50, 100, 200, 400	200	200
Stimulation frequency	-20	100	50, 100, 200	200

Table 3.1: The stimulation parameters used for each experiment. Each stimulation trial was repeated five times.

The preprocessed calcium imaging data was further analysed by checking if the data aligned with the ROIs and corresponding fluorescent traces in the GUI of Suite2p using Python programming language and the output files of Suite2p.

To study the spatial and temporal activation patterns of neurons induced by electrical microstimulation, next, for each ROI, I calculated the baseline fluorescence intensity (F_0), which was the average calcium signal during the prestimulation period (with a typical duration of 10 sec), then computed the baseline corrected calcium trace for each identified ROI (dF/F_0). To identify neurons (ROIs) activated by electrical stimulation, for each neuron, I compared the mean calcium activity during stimulation to the baseline (pre-stimulation) signal. I applied a threshold which was the average baseline signal + three standard deviations. Neurons which exceeded this threshold during stimulation were considered as activated neurons. For each stimulation session and condition, I calculated the mean calcium signal (dF/F_0) of all neurons both during the stimulation and the poststimulation period. For studying the distance-dependent changes in the calcium activity and the number of activated neurons, distance between the centre of the stimulating microelectrode and the centre of each ROI was also determined.

Chapter 4.

Results

In our experiments, we studied the spatio-temporal dynamics of neural activation patterns elicited by controlled electrical microstimulation using flexible polymer-based multi-shank probes in the primary visual cortex of transgenic GCaMP6f and GCaMP6s mice³. We focused on three main parameters that influence neuronal activation: current amplitude, pulse duration and stimulation frequency (Table 3.1). By capturing and analysing calcium signals, we were able to look into neuronal activation dynamics (Fig.4.1).

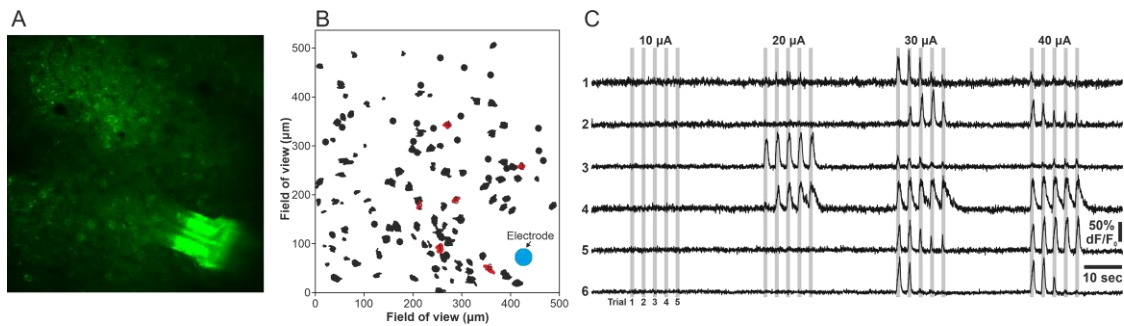


Figure 4.1: Examples of calcium imaging and its analysis in a mouse expressing GCaMP6f. (A) A sample frame of calcium imaging from the visual cortex of an anaesthetised GCaMP6f mouse during electrical stimulation. (B) Regions of interest (ROIs, black and red) identified by Suite2p in the calcium data (A), with the blue ROI indicating the location of the stimulating microelectrode. (C) Baseline-corrected calcium traces of six ROIs (neurons) extracted from the calcium data shown in (A). Calcium traces correspond to ROIs coloured red in (B). Grey vertical bars indicate the stimulation periods.

4.1. Neural responses to electrical stimulation using different current amplitudes

Figure 4.2 highlights the main findings regarding the neural responses in GCaMP6f and GCaMP6s mice to different electrical stimulation amplitudes shown in Table 3.1. Notably, GCaMP6s mice (indicated by red and purple dashed lines) display a significantly higher number of active neurons at each current level compared to GCaMP6f mice (solid lines), indicating a more robust response in GCaMP6s mice. The mean fluorescence intensity (dF/F_0), representing calcium signal strength, also increases with stimulation current for both types of mice (Fig.4.2 B), GCaMP6s mice consistently show higher dF/F_0 values than GCaMP6f mice at equivalent currents, suggesting stronger calcium signalling. While GCaMP6s mice maintain higher

³ Nguyen, E., Horváth, C., Stelcz, R., Rácz, M., Ceyssens, F., Schelles, M., Kraft, M., Ulbert, I., Wittner, L., Fiáth, R. Effect of electrical microstimulation parameters on in vivo neuronal calcium responses in the visual cortex of mice., FENS 2024

fluorescence levels throughout, in both cases, a decrease in mean fluorescence intensity across successive stimulation trials can be observed (Fig.4.2 C).

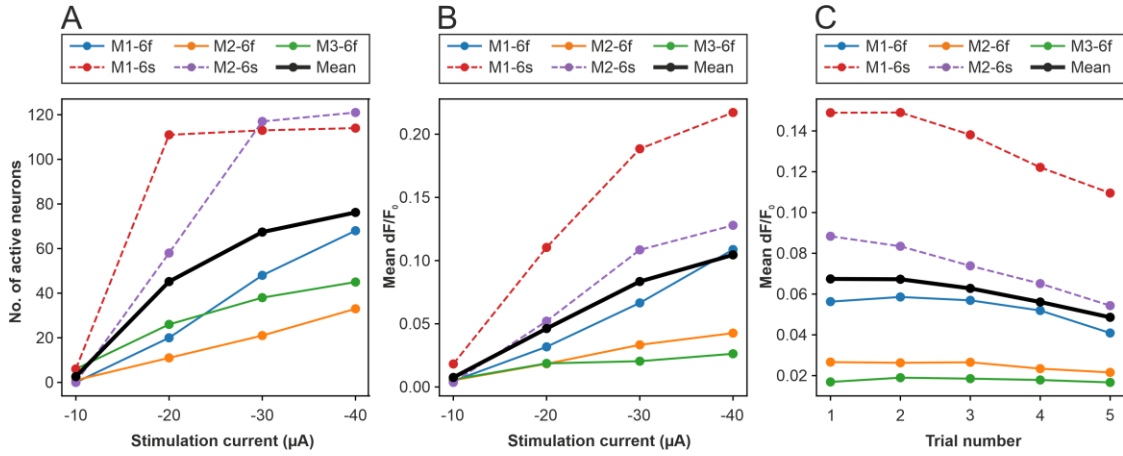


Figure 4.2: Comparison of neuronal activation in GCaMP6f (n=3) and GCaMP6s (n=2, dashed lines) mice under different stimulation amplitudes. (A) Number of activated neurons at different current amplitudes. (B) Mean dF/F_0 across current amplitudes. (C) Mean dF/F_0 over consecutive stimulation trials. The black trace shows the average of the five animals.

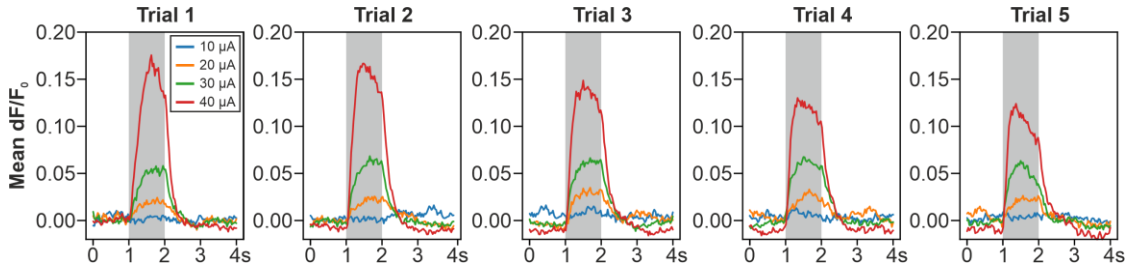


Figure 4.3: Mean calcium traces (dF/F_0) of all identified ROIs (neurons) for each current amplitude (coloured traces) and each trial (1 to 5, left to right) in mouse M1-6f in Fig.4.2. The highlighted grey areas mark the stimulation periods.

To highlight the finer details of neuronal calcium responses to electrical stimulation over time, the mean calcium traces (dF/F_0) of all identified neurons in mouse M1-6f are shown in Figure 4.3, captured across the five consecutive stimulation trials with varying current amplitudes.

Across all trials, there is a clear increase in calcium signal intensity with rising current amplitude (Fig. 4.3). The highest current amplitude (40 μ A, in red) consistently produces the strongest fluorescence response in each trial, with a higher peak and wider signal width compared to lower amplitudes. At 10 μ A (blue trace), the response is minimal, barely deviating from the baseline, indicating that this current level is likely insufficient to generate a robust activation in our mouse model.

The peak fluorescence signal decreases progressively across trials, with the most notable response observed in trial one (Fig. 4.3). By the fifth trial, the overall intensity for each current amplitude appears lower than in the initial trials. The calcium signal rises sharply at the start of each stimulation period and returns to baseline shortly after the stimulation ends.

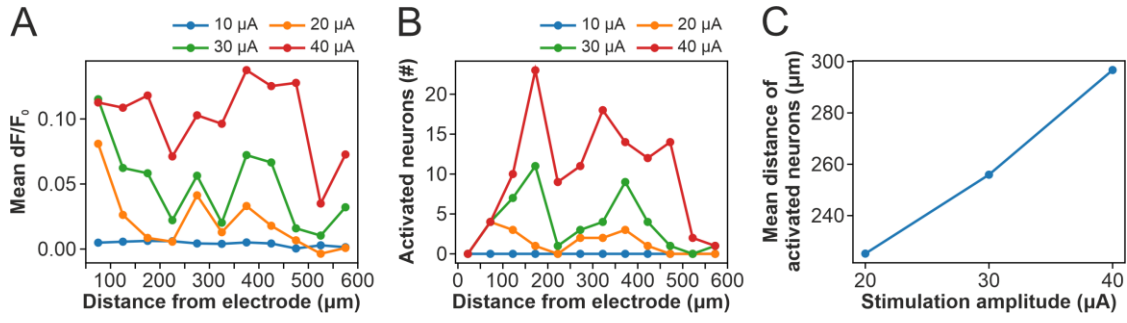


Figure 4.4: Distance-dependent changes in the mean fluorescence (A) and the number of activated neurons (B) during electrical stimulation with different current amplitudes in mouse M1-6f in Fig.4.2. (C) Stimulation amplitude-dependent changes in the mean distance of activated neurons relative to the location of the stimulating microelectrode. No ROIs were identified within 50 μm distance from the electrode and no neurons were activated during 10 μA stimulation.

The distance-dependent effects of cortical microstimulation on neuronal activation in mouse M1-6f can be inspected in Figure 4.4. Mean fluorescence (dF/F_0) representing calcium signalling strength, varies with both distance from the electrode and the stimulation amplitude. The signal is strongest close to the stimulating electrode, however, at the highest stimulation current (40 μA), fluorescence remained elevated across all distances. Lower current amplitudes (10 μA and 20 μA , shown in blue and orange) produce minimal fluorescence, primarily limited to regions closer to the stimulating electrode (Fig.4.4 A). The number of activated neurons peaks between 100-200 μm from the electrode, although at 40 μA , activation extends to more distant regions up, to 600 μm (Fig.4.4 B). For lower current amplitudes (10 μA and 20 μA), the number of activated neurons is minimal.

A summary of the distance relationship with current amplitudes can be observed in Fig.4.4 C. As the stimulation amplitude increases from 20 μA to 40 μA (for 10 μA there were no activated neurons), the mean distance of activation steadily grows.

4.2. Neural responses to electrical stimulation using different pulse durations

The impact of varying pulse durations on neuronal activation and fluorescence intensity (dF/F_0) in GCaMP6f and GCaMP6s mice, with the stimulation parameters in Table 3.1 is demonstrated in Figure 4.5. The number of active neurons increases with pulse duration in both groups, with GCaMP6f mice, particularly mouse M1-6f (blue), showing a marked rise, especially at the longest pulse duration of 400 μs . GCaMP6s mice (dashed lines) also show enhanced activation with longer pulse durations, though the effect is less pronounced than in M1-6f (Fig.4.5 A).

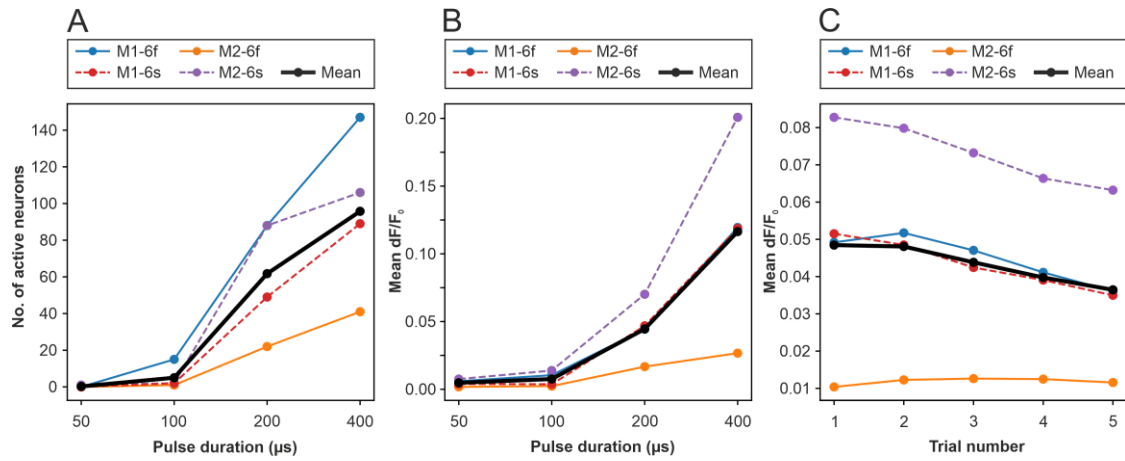


Figure 4.5: Effect of pulse duration on neuronal activation and fluorescence intensity in GCaMP6f (n=2) and GCaMP6s (n=2, dashed lines) mice (A) Change in the number of activated neurons. (B) Change in the mean fluorescence intensity (dF/F_0) (C) Mean dF/F_0 shifts across consecutive trials. The black trace shows the average of the four animals.

A similar trend in mean fluorescence intensity, with dF/F_0 rising as pulse duration increases, occurs in Figure 4.5/B. GCaMP6s mice, especially M2-6s, exhibit stronger fluorescence responses at longer pulse durations (Fig.4.5 B).

Both groups experience a decrease in mean dF/F_0 over consecutive trials. While GCaMP6s mice (notably M2-6s) start with higher initial fluorescence levels, they exhibit a gradual decline across trials, whereas GCaMP6f mice maintain relatively stable, but lower responses (Fig.4.5 C). Figure 4.6 illustrates the effects of varying pulse durations on the distance-dependent activation of neurons during electrical stimulation in mouse M1-6f.

With shorter pulses (50 μ s in blue and 100 μ s in orange), the mean fluorescence intensity (dF/F_0) is highest close to the electrode. While at the longest pulse duration (400 μ s, shown in red), fluorescence intensity remains relatively high even at distances further from the electrode (Fig.4.6 A).

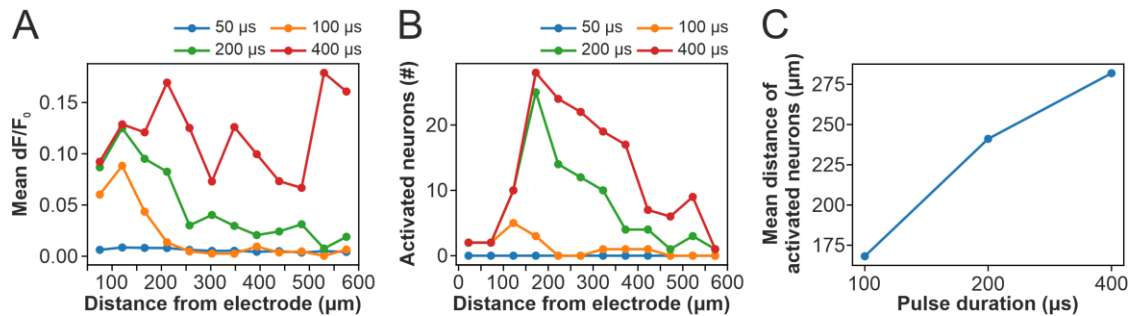


Figure 4.6: Distance-dependent effects of pulse duration in the mean fluorescence (A) on the number of activated neurons (B) with different pulse durations in mouse M1-6f in 4.5. (C) Pulse duration-dependent changes in the mean distance of activated neurons relative to the location of the stimulating microelectrode. No ROIs were identified within 50 μ m distance from the electrode and no neurons were activated with 50 μ s long pulses.

Most activated neurons are located within 100-200 μm from the electrode at these shorter pulse durations. However, with the longest pulse duration (400 μs), the mean fluorescence remains elevated across all distances, showing a relatively uniform response further from the electrode (up to 600 μm). For shorter pulse durations (50 μs and 100 μs), the number of activated neurons are not as outstanding (Fig.4.6 B).

As pulse duration increases from 100 μs to 400 μs (there were no activated neurons with 50 μs) the average distance of activated neurons steadily increases as well (Fig.4.6 C).

4.3. Neural responses to electrical stimulation using different frequencies

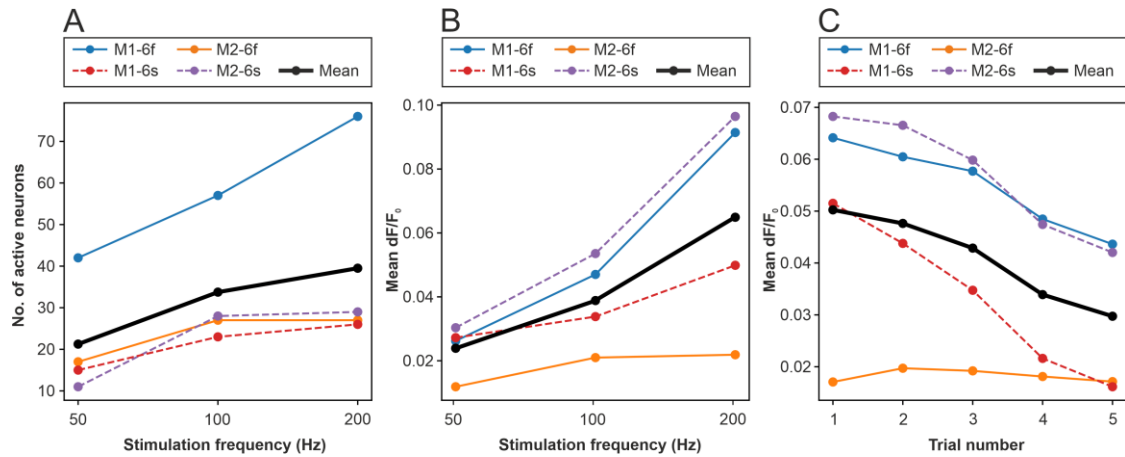


Figure 4.7: Neuronal activation in GCaMP6f and GCaMP6s mice across different stimulation frequencies. (A) Number of activated neurons. (B) Mean fluorescence intensity (dF/F_0). (C) Mean dF/F_0 decreases over consecutive trials.

Figure 4.7 illustrates neuronal activation in GCaMP6f ($n=2$) and GCaMP6s ($n=2$, dashed lines) mice at varying stimulation frequencies, with the stimulation parameters shown in Table 3.1

The number of active neurons increases with stimulation frequency in both types of mice, with GCaMP6f mice (solid lines) exhibiting a steeper rise in neuron activation compared to GCaMP6s mice (dashed lines). Notably, mouse M1-6f (blue) in the GCaMP6f group demonstrates the highest number of active neurons across frequencies reaching ~ 70 neurons at 200 Hz, while mouse M1-6s (red) in the GCaMP6s group shows a more subtle increase (Fig.4.7 A).

A similar increasing trend can be described for the mean fluorescence intensity (dF/F_0), however, a GCaMP6s mouse (M2-6s, purple) exhibits the highest values closely followed by mouse M1-6f (Fig.4.7 B). A decline in mean dF/F_0 across consecutive trials in both groups is also clear from Fig.4.7 C. In GCaMP6f mice, the mean dF/F_0 decrease is more gradual, with higher initial values and a slower decline. GCaMP6s mice (dashed lines) start with higher

fluorescence levels but experience a sharper drop-off by the fifth trial. The distance-dependent effects of different stimulation frequencies on neuronal activity in M1-6f are illustrated in Figure 4.8.

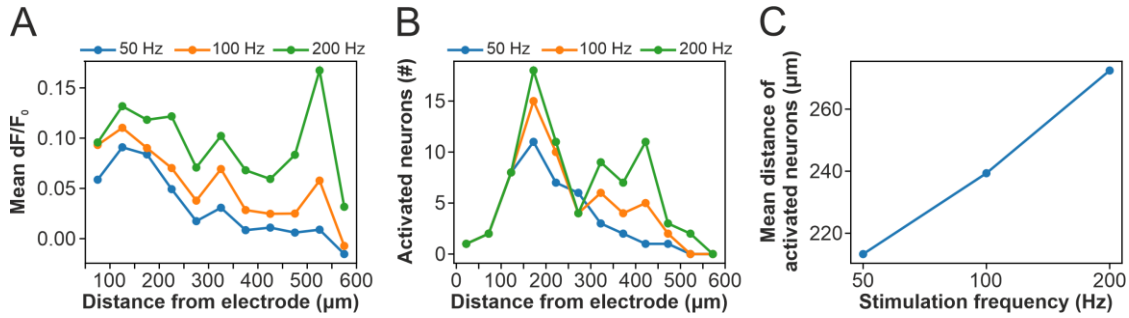


Figure 4.8: Distance-dependent changes in mean fluorescence intensity (A) and the number of activated neurons (B) during electrical stimulation at different frequencies in M1-6f in 4.7. (C) Frequency-dependent shifts in the average distance of activated neurons from the stimulating microelectrode.

For all frequencies, the mean fluorescence intensity (dF/F_0) is strongest near the electrode, with higher frequencies resulting in greater fluorescence overall (Fig.4.8 A). At the highest frequency (200 Hz, shown in green), there is generally higher fluorescence intensity at greater distances from the electrode compared to lower frequencies (50 Hz in blue and 100 Hz in orange). Generally, lower frequencies (50 Hz and 100 Hz) show more localized peaks closer to the electrode. Most activated neurons are located 100-200 μm from the electrode; however, at higher frequencies, a notable number of neurons are activated further from the electrode (up to 500,μm). In contrast, at 50 Hz and 100 Hz, neuron activation is more concentrated near the electrode and decrease quickly with distance (Fig.4.8 B). As the frequency increases from 50 Hz to 200 Hz, the average distance of activated neurons increases linearly, reaching approximately 275 μm at 200 Hz (Fig.4.8 C).

4.4. Neural responses to electrical stimulation to dynamic current steering

The dynamic shifting of current is designed to steer the activation across a spatial gradient (decreases on one stimulation site and increases on the other (Fig. 4.9 B), allowing for the examination of how neurons at different proximities to each electrode respond to the current as it transitions between the two tip electrodes (Fig. 4.9 A).

Calcium activity for neurons, ordered by the latency of their peak response ($-40 \mu A$ in 4.9 C1 and $-50 \mu A$ in Fig. 4.9 D1) can be seen as grayscale heatmaps. The colour gradient in the heatmaps represents the level of calcium response, with lighter colours indicating higher activity. The start of the stimulation is marked by a dashed vertical line. Similar patterns of activation were observed for the two tested stimulation currents.

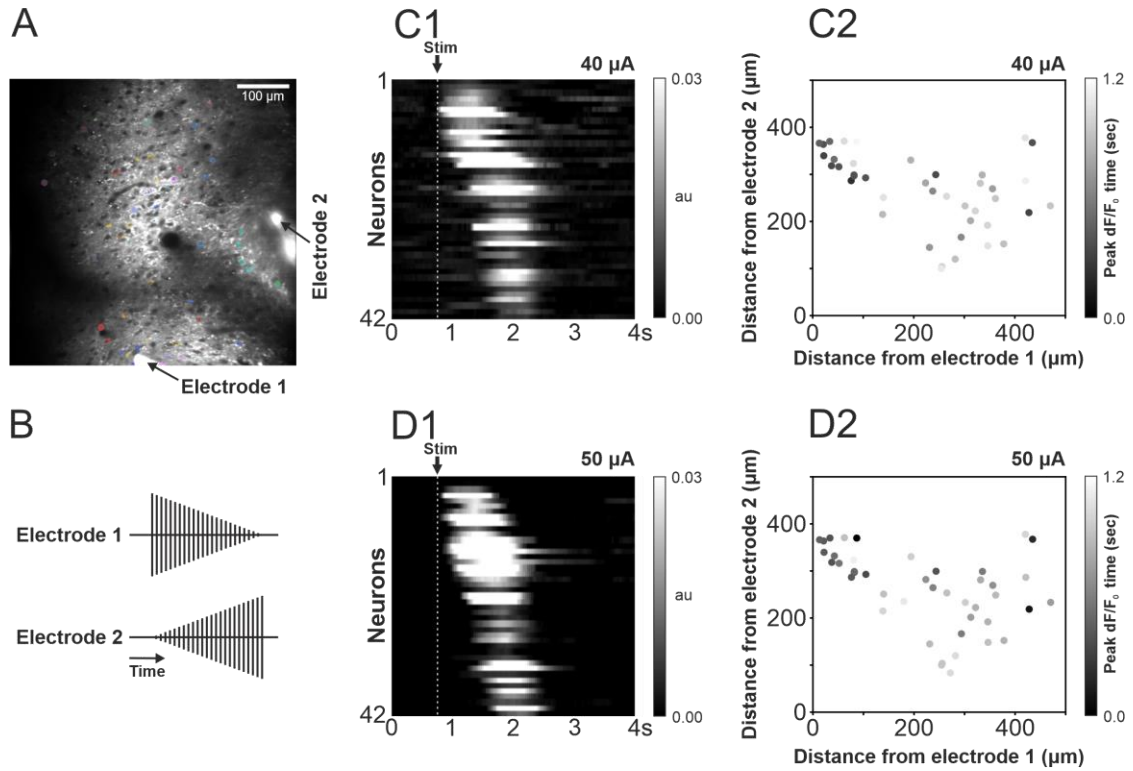


Figure 4.9: (A) Spatial overview, showing the positions of the two electrodes (Electrode 1 and Electrode 2) within the visual cortex of the mouse. The neurons marked around the electrodes indicate the stimulated region. (B) Stimulation pattern between the two electrode tips (C1-D1) Calcium activity of neurons ordered by the latency of their peak calcium response during $-40/-50 \mu\text{A}$ (maximum current) stimulation. (C2-D2) Location of neurons relative to the two stimulation microelectrodes. Color-coding shows the latency of the peak amplitude of the neurons relative to the start of the stimulation.

At $40 \mu\text{A}$, neurons closer to Electrode 2 show delayed peak responses, suggesting a spatialtemporal pattern where neurons nearer the active electrode (as current shifts) respond sooner. Similarly, at $50 \mu\text{A}$, neurons near the stimulated electrode respond with minimal latency, while those further from the electrode peak later.

The scatter plots (Fig. 4.9 C2 and D2) show the spatial relationship between neuron locations and their peak response latencies relative to the two electrodes. Neurons located closer to Electrode 1 (starting the stimulation with higher amplitude pulses) were activated earlier as opposed to neurons located closer to Electrode 2 (providing higher amplitude pulses at the end of stimulation). Neurons located between the two electrodes showed mixed peak latencies. We observed similar propagation patterns to dynamic current steering in multiple mice (data not shown) indicating the reproducibility of these patterns.

Chapter 5.

Discussion

In this study, we investigated how varying electrical stimulation parameters current amplitude, frequency, and pulse duration affect neuronal activation in the visual cortex of anaesthetised GCaMP6f and GCaMP6s mice. This section serves to elaborate on the results and compare our findings with previous research.

5.2. Spatial spread of the neuronal response to electrical microstimulation

Looking at the spatial aspect in relation to activation patterns elicited by different current amplitudes across all measured distances indicates that stronger stimulation can engage neurons farther from the electrode. This broad activation effect aligns with the observation of Histed and colleagues [41] that higher currents activate larger number of neurons across a wider spatial distribution. In contrast, low-amplitude stimulation is insufficient for widespread neuronal engagement. This could be caused by the insertion-related mechanical damage to the tissue around the implant, as well as lower current amplitudes not being sufficient enough to activate neurons in close proximity to the electrode. Similarly, with shorter pulse durations, detected fluorescence was highest near the electrode, indicating localised response, whereas with longer pulses a broader spread of activation can be executed (Fig.4.6 A, C), thus expanding the spatial range of active neurons, suggesting that longer pulses are to some extent beneficial for inducing a widespread activation as short pulses lack the needed intensity to engage neurons located at greater distances. Our experiments with varied stimulation frequencies show analogous trends to the ones with different current amplitude and pulse duration, although not as pronounced as previous findings. With higher frequencies, a more dispersed calcium response across a wider neuronal range can be generated. This extended spatial reach suggests a more robust electric field effect, enabling activation of neurons located further from the electrode.

Our findings align with prior findings published by Michelson and colleagues [57], who demonstrated on anaesthetised GCaMP6s mice that higher stimulation frequencies and extended pulse widths yield increased neuronal activation and broader spatial engagement, especially when targeting cortical regions with electrical microstimulation. They also observed a frequency-dependent enhancement in neuronal recruitment, noting that higher frequencies facilitate sustained responses in proximal neurons while also recruiting distal populations.

5.3. Temporal effects of electrical microstimulation

In all cases, a gradual attenuation in mean fluorescence (dF/F_0) intensity across consecutive stimulation trials (Figs. 4.2, 4.5, 4.7 C) was observed, suggesting an adaptation or desensitization of neuronal responses with repeated stimulation.

This observed pattern suggests possible network limitations, potentially due to synaptic depression or inhibitory mechanisms. The progressive decline in peak calcium response present for all investigated stimulation parameters-could be an indicative of reduced sensitivity over time, with shortened peak response latency and a slight post-stimulation undershoot at higher currents, longer pulse durations and higher stimulation frequencies.

5.4. Comparison of the neuronal responses between GCaMP6f and GCaMP6s mice

It is important to note that the enhanced response in GCaMP6s mice in Figure 4.2 might be partially attributed to the higher stimulation frequency (200 Hz) used in these experiments compared to the GCaMP6f mouse data collected using 100 Hz stimulation (Table 3.1). This increase in frequency results in approximately two times the charge delivery over the same time frame, raising the probability of reaching the activation threshold across a larger neuron population and leading to more widespread neuronal activation (Fig. 4.2 A). A more rapid decline across trials in GCaMP6s mice compared to GCaMP6f ones in Figure 4.5 C could also be the result of neural fatigue, which may stem from the higher stimulation frequency, since it could affect temporal accumulation and reduce frequency responsiveness (Fig.4.7 B). Another source of the mentioned differences could be due to the fact that in GCaMP6s animals, the indicator is expressed in more cells, therefore it is expected to observe more activated cells. In terms of spatial activation, GCaMP6s mice appear to produce stronger individual calcium responses but activate fewer neurons overall than GCaMP6f mice at equivalent frequencies.

5.5. Propagation of neuronal activity during dynamic current steering

The approach of dynamic current steering allows for a controlled directionality in activation, engaging neurons along a specific path with a temporal sequence, as seen in our findings (Fig. 4.9). By ordering neurons based on the latency of their calcium response peaks, we observed a distinct temporal activation pattern. Neurons closer to the electrode with the initial high-amplitude stimulation exhibited earlier response peaks, while those nearer to the electrode with the increasing amplitude responded later, indicating a progressive shift in activation. This confirms the effect of dynamic current steering, where shifting the current between electrodes influences both the timing and location of neuronal activation [53].

5.6. Comparison to previous work

Our findings align with the work of other scholars in the field. First of all, both GCaMP6f and GCaMP6s mice exhibited a decrease in mean dF/F_0 over consecutive trials, indicating neuronal adaptation to repeated stimulation. This phenomenon has been documented in prior research, where repeated electrical stimulation led to reduced neuronal responsiveness over time [57] [44]. Similarly, Histed et al. [41] observed that continuous microstimulation can lead to adaptation, resulting in diminished neuronal responses. We observed that increasing current amplitude led to a higher number of activated neurons and greater mean fluorescence intensity (dF/F_0) in both GCaMP6f and GCaMP6s mice. This dose-dependent relationship is consistent with the findings published by Michelson et al. [57], which concluded that higher current amplitudes result in more extensive neuronal activation.

Furthermore, we observed that longer pulse durations resulted in increased neuronal activation and higher mean fluorescence intensity. This finding is consistent with a previous study that has shown that longer pulses can activate a broader population of neurons (Michelson et al.) [57]. In this study the research group found that higher stimulation frequencies increased the number of activated neurons as well as extended the spatial reach of activation. In our analysis we also noticed a correlation between higher frequencies enhancing neuronal activation and influencing the spatial distribution of active neurons, however, this did not stand out significantly.

As for dynamic current steering, this technique is used in sensory prosthetic applications where precise control over spatial activation is critical for improved resolution and functional outcomes. Our results align with findings by Meikle et al. [53], who demonstrated that intracortical current steering could modulate the location of neuronal activation in a controlled manner, enhancing spatial specificity in neural responses. In the auditory field, studies such as those by Wilson and Dorman (2008) [58] demonstrated that current steering enhances the specificity of auditory nerve stimulation, enabling better frequency selectivity and improving the clarity of sound perception in cochlear implants. Similar to our observations, they found that current steering could shift the centre of activation gradually along an electrode array, achieving a more nuanced control over the neural response. However, the activation was not perfectly sequential, as complex neuronal networks and overlapping dendritic and axonal fields introduced variability. Our findings align with prior research indicating that axonal projections and dendritic projections can introduce unexpected activation patterns, as neurons may respond to stimulation from an electrode distant from their cell bodies due to the proximity of their axons or dendrites to that electrode [59].

The ability to control activation with dynamic current steering has significant implications for neuroprosthetics, where precise spatiotemporal stimulation is essential for creating meaningful sensory experiences. Our findings, along with those from previous studies, suggest that current steering could improve spatial resolution in visual and auditory prosthetics by allowing for finer control over the path and timing of neuronal activation.

5.7. Limitations

5.7.0.1 Indicators and interneuron visualisation

A critical limitation of GCaMP indicators is that they primarily label excitatory neurons, while activity in inhibitory interneurons remains largely undetected. This bias prevents us from obtaining a complete picture of the circuit dynamics in response to electrical stimulation, as interneurons play a significant role in regulating excitatory activity and shaping response patterns. However, there are already research groups looking into this aspect [60].

5.7.0.2 Anatomical differences in the visual system of mouse and primate models

While mouse models provide a useful platform for initial studies, there are notable limitations when translating findings from mouse models to primates or humans. Mouse V1 is relatively simple and lacks the complex hierarchical structure and distinct visual processing streams found in primate visual systems (TOC 2.1.1) [18].

5.7.0.3 Anaesthesia versus awake state

Anaesthesia significantly alters neuronal dynamics, often reducing spontaneous activity and potentially impacting neuronal responsiveness to stimulation. This effect could lead to differences in activation thresholds, adaptation rates, and spatial activation patterns when compared to an awake model. Previous research (Niell, Stryker, 2010)[61] has shown that visual cortical responses differ substantially between anaesthetised and awake states, which may limit the translatability of our findings. Although more challenging, awake, head-fixed mice may provide a more accurate model to examine the spatio-temporal activation patterns in visual cortical neuron networks evoked by electrical stimulation.

5.7.0.4 Constraints of flexible polymer-based probes

Flexible polymer-based probes present a promising advancement in neural interface technology, particularly for chronic implantation. These probes reduce tissue damage compared to traditional rigid electrodes, allowing for better long-term compatibility. However, they come with several limitations, including a more challenging implantation into the brain tissue. Furthermore, these probes can shift within the brain tissue over time, particularly in animal

models that are awake and mobile, complicating consistent targeting of specific neurons or neural populations over time leading to variability in recorded signals and field of view potentially limiting the reliability of this device in chronic applications.

5.8. Future directions

In this thesis, I sought to present the key findings from our study, yet given the complexity of this topic and space limitations, a comprehensive exploration of all results was not possible. Beyond the results showcased here, we also investigated additional aspects such as the stability and repeatability of neural responses to stimulation over time, the cortical depth dependency of stimulation, the effects of pulse number, and variations in neuronal responses between cathodic-first and anodic-first pulses.

In the near future, we plan to utilize awake, head-fixed mice to better capture naturalistic neuronal responses under conditions closer to physiological states. This approach may provide more relevant insights into the spatio-temporal activation patterns in the visual cortical neuron network evoked by electrical stimulation.

Additionally, we plan to further explore neuronal activation patterns using dynamic current steering. By investigating the impact of different stimulation configurations of cortical neuronal activation, we aim to optimize stimulation patterns that can evoke more detailed percepts, which are essential for improving the resolution of visual prosthetics. The parameter space for electrical stimulation is extremely large, making it unlikely to identify an optimal stimulation parameter set solely through in vivo animal experiments in a reasonable timeframe. Consequently, computational modelling and artificial intelligence are expected to play critical roles in the quest for advanced stimulation patterns. As part of the HYPERSTIM project, we have already collaborated with a research partner to develop a precise computational model to study the effects of electrical stimulation in silico.

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